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Study on sex steroid-binding proteins (with emphasize on 17β-estradiol) in plasma of female and juvenile kutum (*Rutilus frisii kutum*)

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Abstract: A sex steroid-binding protein (SBP) that binds to 17β-estradiol with high affinity and moderate capacity was identified in the plasma of female and juvenile of kutum (*Rutilus frisii kutum)* sampled during the early stage of gonadal maturation in October and prior to spawning in March.

Mean maximum specific binding (B_{max}) and equilibrium dissociation constant (K_d) of the fish were as follows: In early stage of gonadal development (October), B_{max} = 5800±970fmol E_2/mg protein, K_d 4.1±0.6nM, and prior to spawning (March) B_{max} = 4000±895fmol E_2/mg protein, K_d 2.9±0.3nM. In juvenile sampled in October B_{max} = 1600±130fmol E_2/mg protein, K_d 2.1±0.2nM and in March samples B_{max} = 3500± 780fmol E_2/mg protein, K_d 2.2±0.2nM. Plasma estradiol binding characteristics of the adult samples in October and March differed from the juvenile samples in having both B_{max} and K_d significantly higher than juveniles. Plasma SBP levels displayed a moderate correlation with GSI (r^2 = 0.52) and CF (r^2 = 0.51) and a weak correlation with HSI (r^2 = 0.28). Affinity was moderately correlated with CF (r^2 = 0.68) and HIS (r^2 = 0.50). A strong correlation was obtained between B_{max} and K_d, high B_{max} values coincided with high K_d values and vice versa.

Keywords: 17β Estradiol, Plasma, Sex steroid-binding protein, Kutum

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Introduction

Bound to a sex steroid binding Protein (SBP), 17β-estradiol (E_2) and testosterone (T) are transported in the plasma of many species. Binding to SBP protects steroids from metabolism. The metabolic clearance rate of T, for example, is reduced in the presence of SBP (Øvrevik *et al.*, 2001). As well as protecting steroids in the plasma, SBPs may also act to control steroid entry to target cells. Specific cell surface receptors have been found for the protein in target areas, although only unliganded SBP can bind to the receptor, steroid can then bind to the SBP–receptor complex. SBP has also been found inside cells, although it is not certain whether this SBP is of extra- or intracellular origin (Hobby *et al.*, 2000).

In most vertebrates, circulating steroids like 17β-estradiol (E_2) and testosterone (T) are transported in the bloodstream bound to specific high affinity sex steroid-binding proteins (SBPs), also known as sex hormone-binding globulin (SHBG) or testosterone-Estradiol-binding globulin (TeBG), and lower-affinity proteins like corticosteroid-binding proteins (CBPs) and albumin (Rosner, 1991; Baker, 1998). Although the function of plasma SBPs is not completely understood, they are believed to protect circulating steroids from rapid metabolic degradation and excretion (Plymate *et al.*, 1990) and to modulate steroid availability and distribution (Rosner, 1991). Recently, a specific SBP cell membrane receptor (RSBP) has been identified, indicating that SBPs are involved in intracellular signal transduction (Rosner *et al.*, 1999). At present, plasma SBP is identified in most vertebrates except birds and some mammalian species (Westphal, 1986).

Kutum is one of the most popular and commercial fish in the Caspian Sea, whose stocks have decreased during the last decade (Kousha *et al.*, 2004). Although several aspects of kutum biology have been examined in Iran with support of Fisheries Organization for increasing its stocks, there is a lack of knowledge on the reproductive endocrinology of this species. This study is the first study on sex steroid-binding protein (SBP) in plasma of kutum. The main objective are on preliminary characterization of sex steroid-binding protein (SBP), SBPs binding activity in female and juvenile of kutum and, finally, find correlation with

some reproductive indices and the effect of heat treatment on specific binding of 17β-estradiol.

Materials and Methods

Chemicals:

The test compounds 17β-estradiol (E_2) was from Sigma (St. Louis, MO), whereas [2, 3, 6, 7-^{3}H] Estradiol ([^{3}H] E_2) was obtained from Nycomed Amersham plc (Buckinghamshire, England). The test chemicals were of highest commercial purity (97%). Prior to use, all test chemicals diluted in methanol and stored at -80°C.

Fish:

Sixty kutum specimens (30 adult females and 30 juveniles) were used in this investigation. Three years old adult females at early and late maturation stages were obtained. The females at early stage of maturation (I and II) with a mean weight of 491±23 (g) were captured directly from the Caspian Sea in October; pre spawning females (stage IV) with a mean weight of 573±14 (g) were obtained from Shirood Hatchery, located at a main natural spawning ground in Mazandaran province in March. Juveniles with a mean weight of 90±9.7 (g) and 110±8.7 (g) were respectively caught in October and March from the Caspian Sea.

Maturity stages were indexed for all groups of kutum following Kesteven scale (1960) with the following modifications: Color, shape and extension of the ovary into the body cavity as well as color and shape of ova were considered to define stage of maturity in females. Degree of transparency of the ovary was also used as a criterion, since it is one of its characteristic features during early as well as fully matures phases.

Kutum brought to the laboratory in open plastic containers (500 lit.) at appropriate temperature; pH 7.0–7.5, and oxygen saturation of 9.5 mg/lit and blood sampling was conducted 24 hours later at 11:00 am.

Blood Sampling:

Blood sampling was conducted in October and March on the early and late maturation stage of adult females and immature juveniles. Prior to sampling, all fish were anesthetized with MS_{222} (200 $mg_{/lit}$) (Sigma). Blood samples were taken

from the caudal artery by heparinized syringes containing 0.1% heparin (5000 IU/ml, Sigma) and 1% of the protease inhibitor aprotinin (10 TIU/ml, Sigma) and centrifuged at 2000*g* (10 min at 4°C) (Øvrevik *et al.*, 2001). The supernatant was carefully decanted and immediately frozen in aliquots at -80°C for subsequent assessment of SBP binding characteristics. Weight and length of the fish samples were taken to calculate the condition factor (CF), (body weight/length^3) × 100; gonadosomatic index (GSI), (gonad weight/body weight) × 100; and hepatosomatic index (HSI), (liver weight/body weight) ×100.

Assay Development:

Plasma steroid binding assessment was based on a modified Dextran Coated Charcoal (DCC) described by Laidley and Thomas (1994). Plasma samples from kutum were thawed and diluted in 50mM phosphate buffer (pH 7.4) containing 0.1% gelatin (PG buffer). Aliquots (200ml) were incubated with tritiated steroid (100ml ([^{3}H] E_2) in the presence (nonspecific binding) and absence (total binding) of a 500- fold excess of unlabeled steroid. Incubation was terminated by adding 200ml ice-cold DCC solution (2.5% charcoal and 0.25% dextran in assay buffer), incubated for 5 min, and centrifuged at 1000*g* for 5 min at 2°C. Each sample was assayed in duplicate or triplicate and kept on ice during all of the above steps. Aliquots of supernatant (300ml) was added to 3ml Ultima Gold liquid scintillation cocktail and counted under standard tritium conditions. The difference between total and nonspecific binding represents the specific plasma steroid binding. Assay conditions (plasma dilution, DCC incubation time, DCC concentration, and centrifugation time and temperature stability) were evaluated and optimal conditions employed in the rest of the analysis.

Characterization of steroid binding activity and heat treatment:

The estradiol binding sites in plasma were characterized by saturation analysis. Plasma samples from female and juvenile kutum were thawed and diluted in PG buffer. Aliquots were incubated (4h at 4°C) with increasing concentrations of [^{3}H] E_2 to demonstrate saturability. Maximum specific binding (B_{max}) and the equilibrium dissociation constant (K_d) were determined by Scatchard analysis and nonlinear regression of the specific binding data (GraphPad Software Inc.).

Association kinetics was examined by incubating plasma samples with [^{3}H] E2 for periods from 30 s up to 24h. For determination of dissociation kinetics, plasma samples were incubated overnight (16h at 4°C) with [^{3}H] E_2 and then incubated with a 500-fold excess of unlabeled 17β-estradiol for periods of 30 s to 24 h. Binding specificities were assessed under competitive conditions by incubating diluted plasma samples overnight (16h at 4°C) with 2.5nM [^{3}H] E_2 in combination with increasing concentrations of different unlabeled endogenous hormones. Specific binding of [^{3}H] E_2 was quantified as described previously, and calculated as the binding of [^{3}H] E_2 in the presence of test compounds relative to the control (2.5% vehicle methanol alone) (Øvrevik *et al.*, 2001; Laidly & Thomas, 1994).

IC_{50} values of the test compounds were calculated as the concentration causing 50% inhibition of [^{3}H] E_2 binding. Relative binding affinity (RBA) was determined by comparing the obtained IC_{50} value (IC_{50}-ligand) with that of 17 β-estradiol (IC_{50}- E_2). Furthermore, the equilibrium inhibition constant (K_i) and relative binding affinity (RBA) were calculated as described by Bylund (1980) according to these formulas:

$K_i = IC_{50}/(1 + F/K_d)$

Where, F is the concentration of unbound (free) ligand.

RBA = (IC_{50}-E_2/IC_{50}-ligand) × 100

Specific binding of 17β-estradiol in diluted plasma (0.2mg protein/ml) from female kutum was also determined in different temperature (0°C-60°C) by using heat incubation and subsequent cooling on ice.

Protein Content:

Plasma protein content was determined by the method of Bradford (1976), after modification for micro titer plates. In summary, a protein dye solution (Bio-Rad Laboratories, Hercules, and CA) was diluted in distilled water (1:5) and filtered (Whatman No.1). Samples were diluted in 50mM phosphate buffer (gelatin-free PG buffer), applied in triplicate (10ml) to micro titer wells, and added the protein dye working solution (200ml). After 5 min at 20°C, the absorbance was measured (590nm) and protein content determined with the use of bovine serum albumin as protein standard.

Statistical Analysis:

All statistical analyses were performed in Mini tab and data are presented as means ±SEM. Differences between groups were analyzed by one-way ANOVA for normally distributed data or Kruskal–Wallis nonparametric test when data failed tests of normality. Comparison between individual samples (juvenile and female) was made by t Test, whereas relations between parameters (K_d, B_{max}, GSI, HSI, and CF) were examined by linear regression analysis.

Results

Assay Development:

Heat incubation and subsequent cooling on ice resulted in reduced [^{3}H] E_2 binding at temperatures above 25°C, whereas incubation at temperatures above 50°C resulted in total loss of specific binding (Fig. 1).

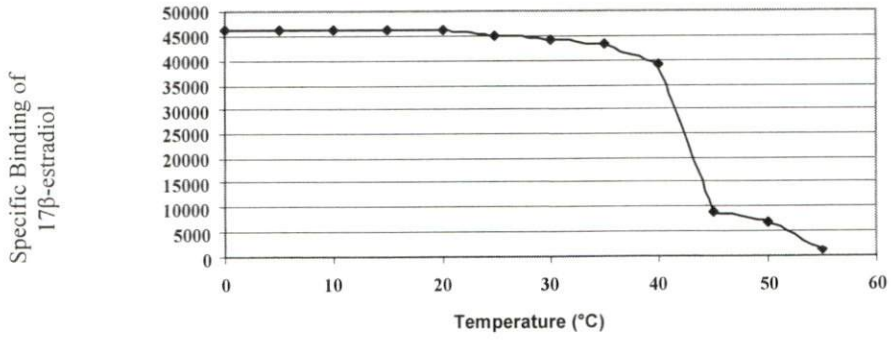

Figure 1: Effect of heat treatment on specific binding of 17β-estradiol in diluted plasma (0.2mg protein/ml) from female kutum. The points depict means of triplicates from one representative experiment (SEM are insignificant).

Characterization of steroid binding activity:

Diluted plasma from kutum was found to bind [^{3}H] E_2 in a specific and saturable manner (Fig. 2).

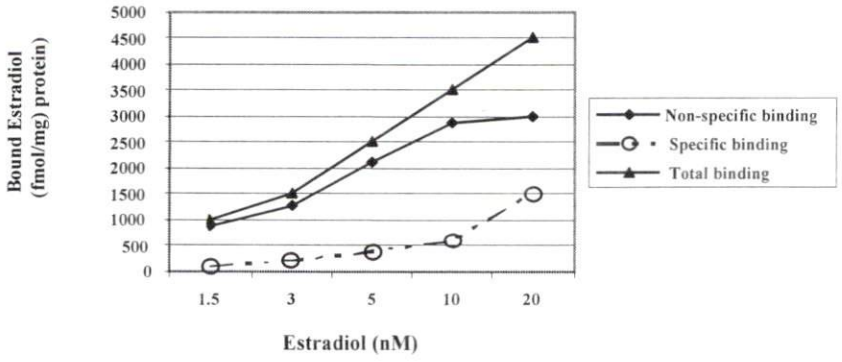

Figure 2: Total, non-specific and specific binding of tritiated 17β-estradiol in diluted plasma (0.2 mg protein/ml) from kutum. The points depict means of triplicates from one representative experiment (SEM are insignificant).

The presence of a distinctive class of binding sites with high affinity and moderate capacity was confirmed by scatchard transformation of the specific binding data (Table 1).

Table 1: Equilibrium inhibitory concentration (IC_{50}), equilibrium inhibition constant (K_i), and relative binding affinity (RBA) for 17β-estradiol to the plasma high-affinity estradiol binding site in kutum.

Hormone	17β-estradiol
IC_{50} (mol/lit)	2.19×10^{-9}
K_i (mol/lit)	8.75×10^{-10}
RBA (%)	100

Mean maximum specific binding (B_{max}) and equilibrium dissociation constant (K_d) of fish sampled were as follows: In adult females, early stage of gonadal development (October), B_{max} = 5800±970fmol E_2/mg protein, K_d 4.1±0.6nM and prior to spawning (March) B_{max} = 4000±895fmol E_2/mg protein, K_d 2.9±0.3nM. In juvenile sampled in October B_{max} = 1600±130fmol E_2/mg protein, K_d 2.1±0.2nM, and in March sample B_{max} = 3500±780fmol E_2/mg protein, K_d 2.2±0.2nM. Plasma estradiol binding characteristics of the adult kutum samples differed from the juvenile samples in having both B_{max} and K_d significantly higher than juvenile ($P<0.05$), and also females displayed significantly high values of B_{max} prior to spawning and K_d in early stage of gonadal development (Fig. 3). A strong correlation was obtained between B_{max} and K_d (r^2 = 0.85, $P<0.001$); high B_{max} values coincided with high K_d values and vice versa (Fig. 4).

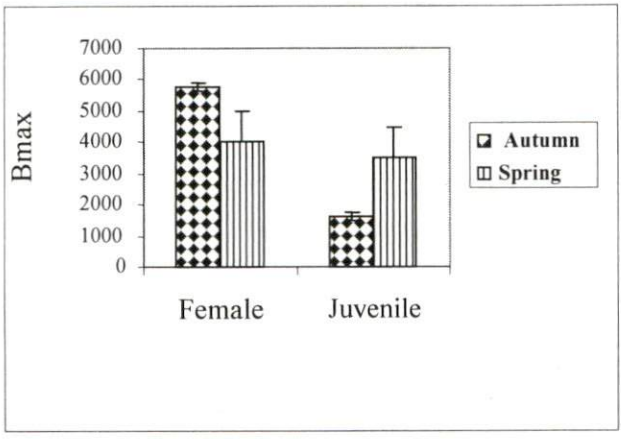

Figure 3: Maximum specific estradiol binding, B_{max} (fmol E_2/mg Protein) of diluted plasma (0.2 mg protein/ml) from kutum sampled during autumn and spring. The results (means±SEM) are based on analysis of 30 individuals in each group.

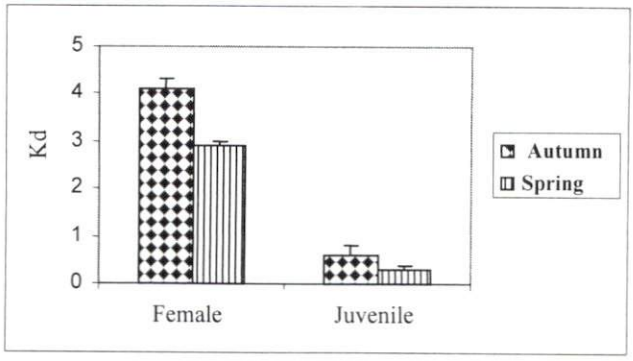

Figure 4: Equilibrium dissociation constant, K_d (nM) from kutum sampled during autumn and spring. The results (means ±SEM) are based on analysis of 30 individuals in each group.

Correlation of SBP with some biometrical factors (Table 2):

Plasma SBP levels displayed a moderate correlation with GSI (r^2 = 0.52, P = 0.03), CF (r^2 = 0.51, P<0.01) and weak correlation with HSI (r^2 = 0.28, P = 0.05). Affinity was moderately correlated with HSI (r^2 = 0.50, P<0.001) and Cf (r^2 = 0.60, P<0.01).

Table 2: The mean (±SEM) HIS, GSI and Cf of juvenile and female of kutum in autumn and spring (n=30 in each group).

	Season	Factor	Mean value (%)
Female (n=30)	Autumn (n=15)	HSI	1. 18
		GSI	2. 04
		Cf	1. 61
	Spring (n=15)	HSI	1. 84
		GSI	9. 87
		Cf	1. 81
Juvenile (n=30)	Autumn (n=15)	HSI	1. 22
		GSI	-
		Cf	1. 63
	Spring (n=15)	HSI	1. 37
		GSI	-
		Cf	1. 55

Discussion

Plasma SBPs have previously been identified in several species including Arctic charr (*Salvelinus alpinus*) (Øvrevik *et al.*, 2001), spotted sea trout (Laidley & Thomas, 1994), Atlantic salmon (Lazier *et al.*, 1985), brown trout (Pottinger 1988), and rainbow trout (Fostier & Breton, 1975), but this is the first report on kutum.

Optimal conditions for the steroid-binding assay was in agreement with Arctic Charr (*Salvelinus alpinus* L.) as cited by Øvrevik *et al.*, (2001) and differed somewhat from those for the assay developed in *Cynoscion nebulosus* as cited by Laidley & Thomas (1994).

There was no effect of DCC exposure time, which contrasts with Øvrevik *et al.*, (2001), Laidley & Thomas (1994) findings, in which this appeared to be critical for the assay, causing a rapid decrease in specific binding during the first 5 min of incubation. However, in accordance with Øvrevik *et al.*, (2001) attempts to strip Arctic charr (*Salvelinus alpinus*) plasma of endogenous steroids, by incubating with DCC prior to assay, did not increase steroid binding and was therefore omitted.

Heat exposure of the kutum SBP like Arctic charr SBP (*Salvelinus alpinus*) (Øvrevik *et al.*, 2001) revealed that the protein was far more heat labile than SBP from the spotted sea trout, *Cynoscion nebulosus* (Laidley & Thomas, 1994). Steroid binding activity of kutum SBP like Arctic charr (*Salvelinus alpinus*)(Øvrevik *et al.*, 2001) was reduced by exposure to temperatures above 25°C (Fig. 1), whereas SBP from the spotted sea trout tolerated short time exposures at temperatures up to 50°C without loss of steroid binding activity (Laidley & Thomas, 1994). This difference is probably due to the fish ambient temperature.

There is a general agreement that some of the main functions of plasma SBPs include protection of circulating steroids from rapid metabolic degradation and excretion and modulation of steroid availability and distribution to target tissues (Rosner, 1991).

Despite a remarkable diversity in steroid specificity among vertebrate SBPs, a relatively high affinity binding of estrogens and androgens and low affinity binding of C_{21} steroids like progesterone and corticosterone seems to be common in fishes (Fostier & Breton, 1975; Laidley & Thomas, 1994). The present analysis reveals that plasma of kutum binds [^{3}H] E_2 with high affinity and moderate capacity (Table 1). The same results have been reported in these species, including Arctic charr (*Salvelinus alpinus*)(Øvrevik *et al.*, 2001), spotted sea trout (Laidley & Thomas, 1994), Atlantic salmon (Lazier *et al.*, 1985), brown trout (Pottinger, 1988), and rainbow trout (Fostier & Breton, 1975).

Females of kutum displayed significantly high values of B_{max} and K_d in early stage of gonadal development (stage I and II) and low levels of them prior to spawning (stage IV), which resembles females of Arctic charr (*Salvelinus alpinus*) that sampled at start and end of gonadal maturation (Øvrevik *et al.*, 2001). The general picture seems to be that plasma SBP levels rise during gonadal maturation in the early and mid reproductive period and subsequently drop in the late reproductive period prior to spawning or birth (Paolucci & Difiore, 1994; Pottinger, 1988; Foucher *et al.*, 1992; Laidley & Thomas, 1997). This subject was proved in Arctic charr (Øvrevik *et al.*, 2001) and was confirmed in kutum females as well.

The results of this study revealed that the levels of K_d and B_{max} in juveniles of kutum changed in autumn and spring and had higher levels in spring. Seasonal variation in plasma SBP levels is reported in a range of vertebrate species including mammals (Schiller *et al.*, 1978; Audy *et al.*, 1981), reptiles (Riley *et al.*, 1988; Paolucci *et al.*, 1992), amphibians (Paolucci & Difiore, 1994), and teleosts (Pottinger, 1988; Foucher *et al.*, 1992; Laidley & Thomas, 1997).

Furthermore there is a strong correlation between B_{max} and K_d values which high SBP levels are associated with low affinity. A similar connection between B_{max} and K_d has previously been observed in the spotted sea trout (Laidley & Thomas, 1997). Several factors are known to affect both plasma SBP levels and affinity

(Rosner, 1991), and the observed connection may thus represent a regulation mechanism or compensatory response where reduced binding affinity is overcome by increased SBP levels, and vice versa.

Considering the strong correlation between B_{max} and K_d, it is difficult to determine the true nature of the correlation between these two parameters and GSI, HSI, and CF. However, a certain correlation between SBP levels and HSI is expected since the liver is suggested to be the main organ for SBP synthesis (Foucher *et al.*, 1991; Hammond & Bocchinfuso, 1995). Furthermore, the correlation between B_{max}, K_d, and GSI is in agreement with the suggestions of a close association between plasma SBPs and the animals' reproductive physiology (Laidley & Thomas, 1997).

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